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Award Number: DAMD17-03-1-0267

TITLE: RNA-Binding Proteins as Novel Oncoproteins and Tumor Suppressors in Breast Cancer

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REPORT DATE: May 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2004	Annual (7 Apr 2003 - 6 Apr 2004)

4. TITLE AND SUBTITLE
RNA-Binding Protein as Novel Oncoproteins and Tumor Suppressors in Breast Cancer

5. FUNDING NUMBERS
DAMD17-03-1-0267

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: ALL DTIC reproductions will be in black and white

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The level of an mRNA depends not only on its rates of synthesis, processing, and transport, but also its rate of turnover. This is particularly important for oncoproteins and cell cycle proteins because their sustained synthesis can favor cell growth rather than differentiation, a hallmark of the neoplastic phenotype. This control is exerted via a balance between the action of at least two RNA-binding proteins, AUF1 and HuR. AUF1 targets the degradation of mRNA like the c-myc proto-oncogene and the cell cycle regulator cyclin D1. By contrast, HuR promotes stabilization of mRNAs. C-myc and cyclin D1 are particularly important in this regard, since both can play a causative role in mammary tumorigenesis. In Phase I, we are examining the effects of AUF1 and HuR expression levels on gene expression in cultured cells. In Phase II, we will assess the roles of AUF1 and HuR in cell growth and tumorigenesis in vivo. During this funding period, we have initiated work on Phase I by creating the expression vectors that will permit either overexpression or knocked-down expression of AUF1 or HuR (e.g. low, medium, or high expression levels) in human breast carcinoma cells. Selection of stably transfected cells has also begun.

14. SUBJECT TERMS
Breast cancer, RNA-binding proteins, AUF1, HuR, mRNA, stability

15. NUMBER OF PAGES
11

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

The level of a messenger RNA depends not only on its rates of synthesis, processing, and transport, but also its rate of turnover. The turnover rate of a mRNA can, in turn, determine its lifetime as a template for protein synthesis. It is particularly important to understand how the levels of mRNAs encoding oncoproteins and cell cycle proteins are regulated because sustained synthesis of these gene products can favor cell growth rather than differentiation, a hallmark of the neoplastic phenotype. Many cell cycle and proto-oncogene mRNAs exhibit extremely short half-lives. Their decay is controlled in part by A+U-rich elements (AREs) located in the 3'-untranslated region. Moreover, the half-lives of their mRNAs are frequently subject to regulatory control. This control is exerted via a balance between the action of at least two ARE-binding proteins, AUF1 and HuR. AUF1 targets the degradation of ARE-mRNAs like the c-*myc* proto-oncogene and the cell cycle regulator cyclin D1. By contrast, HuR promotes stabilization of ARE-mRNAs. c-*myc* and cyclin D1 are particularly important in this regard, since both can play a causative role in mammary tumorigenesis. Our central hypothesis is that AUF1 may act as a novel tumor suppressor by limiting expression of genes that promote cell growth. On the other hand, HuR may act as a novel oncoprotein by stabilizing those mRNAs. Our approach is to alter the expression of AUF1 or HuR in human breast carcinoma cells and examine the resulting effects on cell growth and tumorigenesis in a nude mouse model. Specifically, we have two goals: (1) to examine the effects of AUF1 and HuR expression levels on gene expression in cultured cells; and (2) to assess the roles of AUF1 and HuR in cell growth and tumorigenesis *in vivo*. We plan to introduce into human breast carcinoma cells expression vectors that will permit either overexpression or knocked-down expression of AUF1 or HuR. We will classify clones by their expression levels of AUF1 or HuR (e.g. low, medium, or high). These will then be profiled for the resulting effects on gene expression using a novel DNA microarray strategy. Genes involved in the cell cycle, metastasis and invasion, and angiogenesis will be of particular interest, as many of these contain AREs. Cell clones will also be tested for both their growth phenotypes and their ability to induce tumors as xenographs on nude mice. Our prediction is that tipping the balance to high levels of AUF1 expression will reduce the expression of ARE-mRNAs important for the cell cycle, invasion, metastasis, and angiogenesis. This will likely lower the efficacy of engineered cell lines to induce tumors. By contrast, tipping the balance to high levels of HuR expression will likely increase expression of these genes and lead to increased tumorigenic efficiency.

BODY

The approved SOW is as follows:

Task 1. To examine the effects of AUF1 and HuR expression levels on gene expression in cultured breast carcinoma cells (Months 1-24):

a. Construct Tet-Off plasmids for overexpression of AUF1 and HuR (Months 1-4).
b. Construct plasmids for RNA interference (RNAi)-based knockdown of AUF1 and HuR (Months 1-4).
c. Transfect plasmids into breast carcinoma Tet-Off cells and select individual clones (Months 4-24).
d. Characterize clones for AUF1 and HuR expression levels to define those with low, medium and high levels of AUF1/HuR expression (Months 6-24).
e. Using RNA from engineered cell lines, perform DNA microarray analyses of genes affected by altered AUF1/HuR expression, in particular, those involved in the cell cycle, invasion, metastasis, and angiogenesis (Months 6-24).
f. Identify those transcripts that are direct binding targets of AUF1 and HuR (Months 6-24).

Task 2. To assess the roles of AUF1/HuR in cell growth and tumorigenesis (Months 6-36):

a. Characterize the growth phenotypes of engineered cell lines obtained from Task 1d (Months 6-24).
b. Characterize the cell cycle distribution of these cell lines by flow cytometry (Months 6-24).
c. Introduce clones onto nude mice and score tumor formation as a function of AUF1/HuR expression levels and growth/cell cycle phenotypes (Months 6-36).

We are currently in the midst of Task 1-a, -b, and -c. We have prepared a tet-regulated expression construct for p37AUF1. Figure 1 contains a map of this plasmid. Construction of tet-regulated p40AUF1 and HuR is in progress. For Task 1-b, we have completed preparation of short hairpin RNA (shRNA) vectors for AUF1 and HuR. We accomplished this by annealing oligos containing sequence of the respective mRNAs, a short hairpin, and sequence antisense to the 5' portion of the oligo (Figure 2). We then cloned these into a pSilencer vector (Figure 2). We then sequenced across the length of the inserted oligos to insure fidelity. Figure 3 depicts the sequencing waveform for the HuR shRNA vector and Figure 4 depicts the sequencing waveform for the AUF1 shRNA vector. Specifically, the nucleotides between positions 30 and 99 are the shRNA-encodng bases in Figures 3 and 4. The AUF1 shRNA will knockdown expression of all four AUF1 isoforms. We plan to create vectors for isoform-specific knockdown of AUF1 as well, for comparison. Transfection of the vectors we already have has begun for Task 1-c. We have had a problem, however, in that MCF7-Tet Off cells are quite difficult to transfect. We are trying a new transfection method using Fugene (Roche), which is supposed to be useful for cell lines difficult to transfect. If Fugene reagent does not solve our problem, we will turn to a lentiviral vector system recently shown to permit virtually 100% introduction of shRNA RNAs into cells (J. Virol. 2003 77: 8957-8951). In any event, once we solve this problem, we can begin Task 1-d.

KEY RESEARCH ACCOMPLISHMENTS

- Preparation of pTRE-p37AUF1, an expression construct for overexpression of p37AUF1 in MCF7-Tet Off breast carcinoma cells (Figure 1)
- Preparation of expression constructs for inducible shRNA formation in MCF7-Tet Off cells (Figures 2-4). These will permit tetracycline-induced knockdown of AUF1 and HuR.

REPORTABLE OUTCOMES

We are in the process of building the tools that will allow us to reengineer expression of the key ARE-binding proteins AUF1 and HuR in human breast carcinoma cells. We have prepared three vectors so far. Our feeling is that preparation of vectors does not, per se, constitute a reportable outcome (i.e., a peer-reviewed publication).

CONCLUSIONS

Our central hypothesis is that AUF1 may act as a novel tumor suppressor by limiting expression of genes that promote cell growth. On the other hand, HuR may act as a novel oncoprotein by stabilizing those mRNAs. Our approach is to alter the expression of AUF1 or HuR in human breast carcinoma cells and examine the resulting effects on cell growth and tumorigenesis in a nude mouse model. Specifically, our work has two phases: (I) examining the effects of AUF1 and HuR expression levels on gene expression in cultured cells; and (II) assessing the roles of AUF1 and HuR in cell growth and tumorigenesis *in vivo*. We are currently focused on Phase I. This phase requires us to prepare expression vectors that will permit either overexpression of knocked-down expression of these two proteins. We have about half the required vectors completed. In the meantime, we have begun transfecting these into breast carcinoma cells for stable selection. We have experienced some transfection difficulties, but these are not insurmountable, since we can utilize a lentiviral vector system if need be. These tools (vectors and cell lines) will allow us to address our novel, central hypothesis stated above.

Why is this important? The discovery of AREs in 1986 and the subsequent identification of the ARE-binding proteins that control ARE-mRNA decay have led to the realization that many genes that play active roles in oncogenesis are regulated via their AREs. What has unfortunately received scant attention is the idea that the regulators of these important ARE-mRNAs might themselves possess oncogenic or tumor suppressor activities. Our work is designed to address this idea. Two potential contributions of our studies are: (1) They could have a major impact on our thinking about the pathways by which normal cells become transformed. This is particularly true in breast cancer, since c-*myc* and cyclin D1, two AUF1-regulated mRNAs, are frequently overexpressed. (2) Our studies could spur development of a new generation of pharmaceuticals designed to target an ARE-binding protein such as AUF1 or HuR. This strategy would permit control of large networks of ARE-mRNAs by simply acting on a single target ARE-binding protein.

REFERENCES

None

APPENDICES

Four figures depicting the results of our vector constructions are enclosed as pages 8-11, respectively.

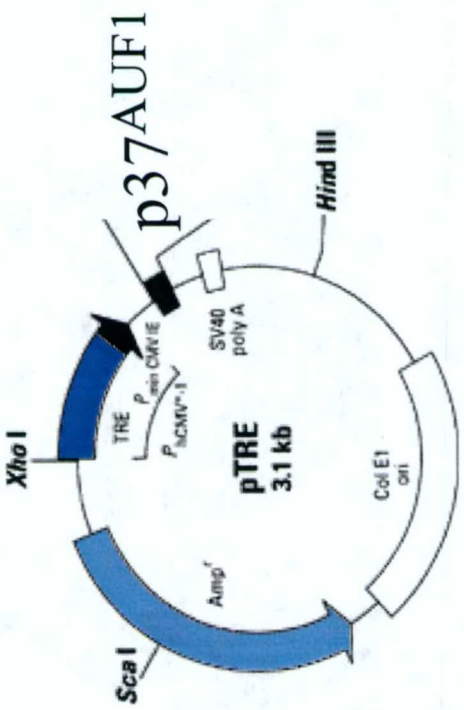
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Figure 1

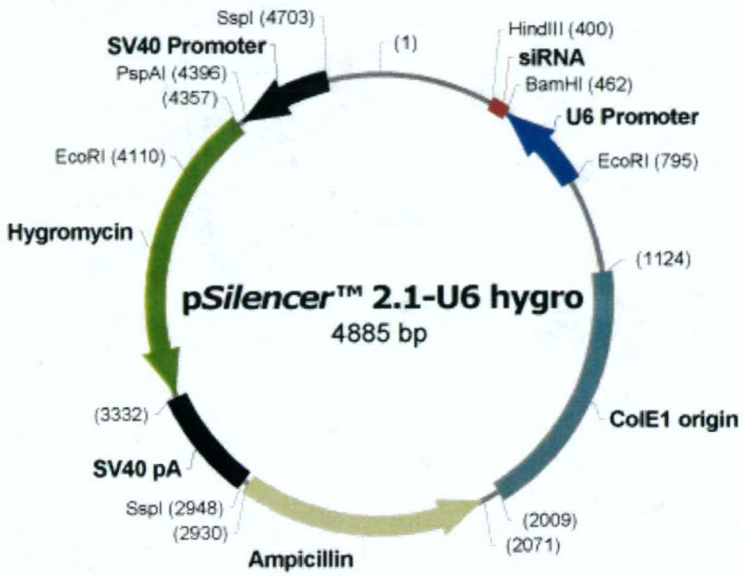

Figure 2

shHuR

BamHI Sense loop Antisense

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GGC*GTGCGACTTGCCGAACTCCA***AGTTCTC***TGGAGTTCGGCAAGTCGCAC*AAAAAACCTTTTCGA-5'

HindIII

shAUF1

BamHI Sense loop Antisense

5'-GATCCC*GTTGTAGACTGCACTCTGAT***TCAAGAG***ATCAGAGTGCAGTCTACAAC*TTTTTTGGAAA-3'

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HindIII

Sh Hu R

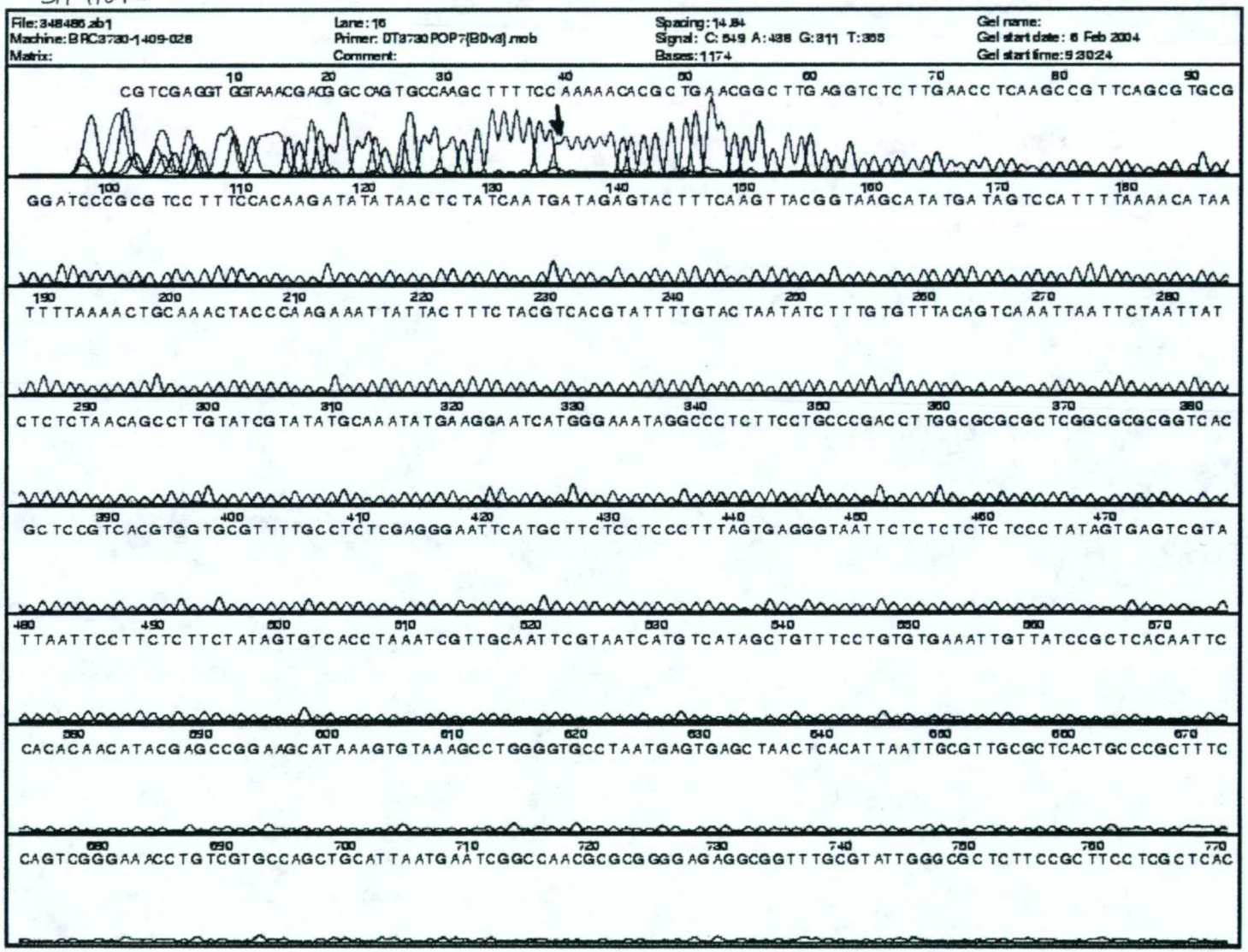
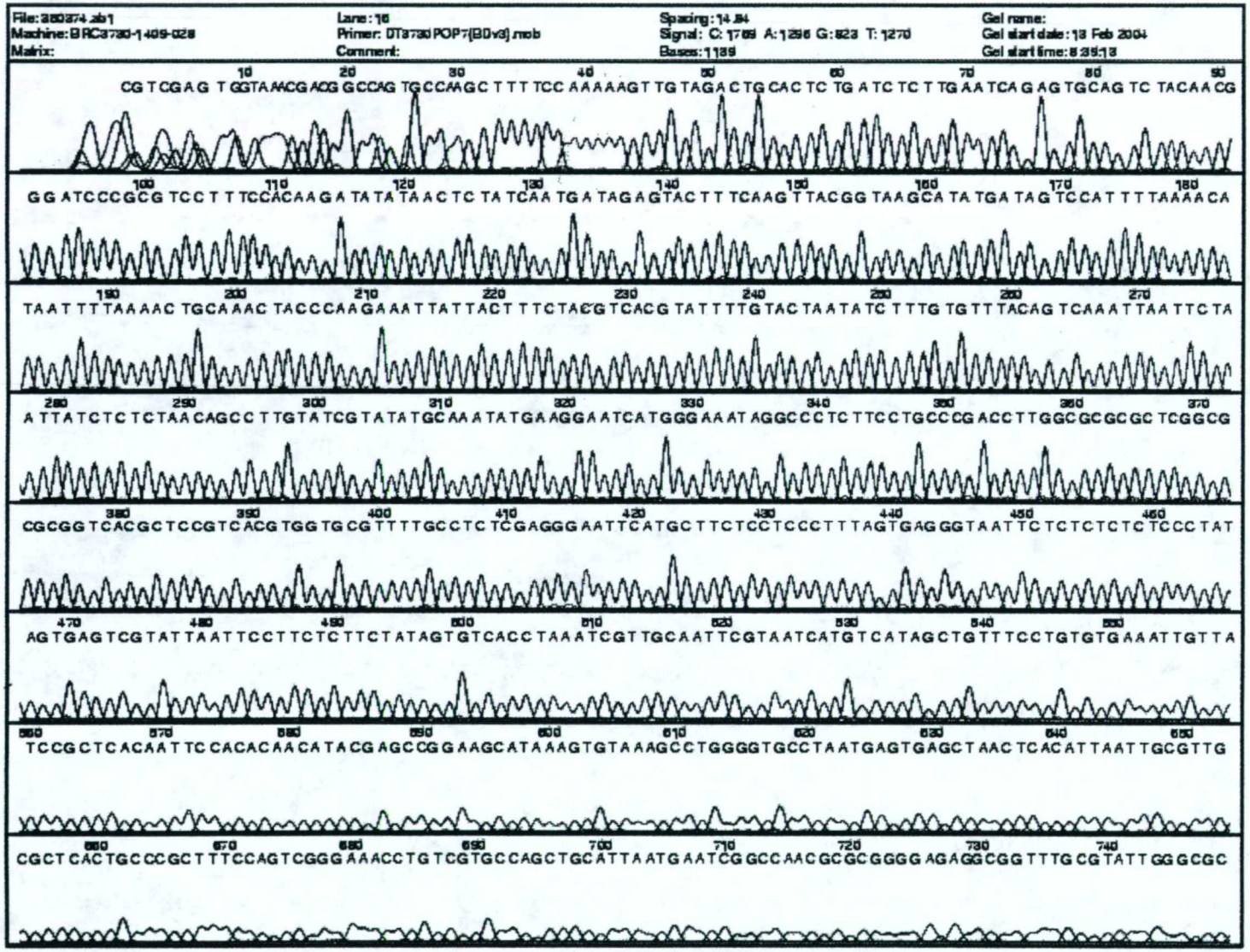


Figure 3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Comment:
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Bases: 1139
Gel name:
Gel start date: 18 Feb 2004
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TAA T TT TAAAAC TGCAAAC TACCCAAGAAAT TA T TACT T TC TACGTCACG TA T T T TG TAC TAA TA TC T T TG TG T T TACAG TCAAA T TAA TTC TA
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CGC T CAC TGCCCGC T T TCCAG TCGGGAAACC TG TCG TGCCAGC TGCA T TAA TGAA TCGGCCAACGCGCGGGGAGAGGCGG T T T G CG TA T T GGGCGC

Figure 4

11